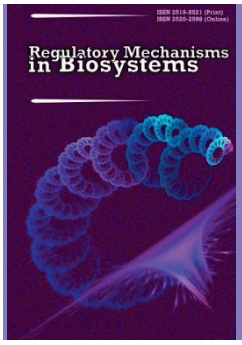

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Low doses of imidacloprid induce neurotoxic effects in adult marsh frogs: GFAP, NfL, and angiostatin as biomarkers

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Imidacloprid is one of the most widely used insecticides in the world. The neurotoxicity of imidacloprid in adult amphibians has not been studied thoroughly. We investigated the expression of glial fibrillary acidic protein (GFAP), neurofilament light chain (NfL) and angiostatin in the amphibian brain to identify valid biomarkers of low dose imidacloprid exposure. For the experiment, 30 individuals of the marsh frog *Pelophylax ridibundus* were selected. The amphibians were divided into five groups. The duration of the experiment was 7 and 21 days. The exposure concentrations were 10 and 100 µg/L. The results of the study revealed a decrease in the expression of GFAP after 7 days in the exposure groups of 10 and 100 µg/L. An increase in the level of NfL was observed in the group exposed to 10 µg/L after 21 days of the experiment. The angiostatin level was increased after 7 days at 10 µg/L and after 21 days at 100 µg/L. The data obtained indicate that low concentrations of imidacloprid can cause neurotoxic effects in the brain of *P. ridibundus*. Such effects can have a significant impact on amphibian populations. According to the results of the study of the expression level of GFAP, NfL and angiostatin, it can be stated that imidacloprid has a neurotoxic effect on adult marsh frogs. The studied indicators can be promising biomarkers of environmental pollution by neonicotinoids.

Keywords: neonicotinoids; insecticides; neurotoxicity; amphibians; brain proteins.

Introduction

More than one third of agricultural products are grown with the use of pesticides (Liu et al., 2002; Zhang, 2018). This helps to reduce economic losses and increase the resistance of crops to pests and adverse climatic conditions. However, despite the use of pesticides, crop losses due to pests are about 40% (IPPC Secretariat, 2021). Given global population growth, pesticide use can be expected to increase in the coming years (Crist et al., 2017). Uncontrolled use of insecticides leads to significant environmental disruption (Udeigwe et al., 2015). Insecticide residues accumulate in the soil and are washed away by surface runoff into surrounding water bodies. Due to the increase in local concentrations of insecticides in water bodies, the risks of toxic effects on non-target organisms are significantly increased (Martynov et al., 2019; Zikankuba et al., 2019; Kozak et al., 2020). The use of insecticides should be environmentally sound. Therefore, research on the biological and environmental consequences of the use of these substances is extremely important (Popp et al., 2013; Yesipova et al., 2022).

Imidacloprid is resistant to photolysis, hydrolysis and elevated temperatures in the environment. The half-life of these substances under certain conditions can be up to 1000 days. This leads to their accumulation during repeated use (Bonmatin et al., 2015). Neonicotinoids disrupt nervous system functions by interfering with neural impulse transmission. These substances affect the functional state of the vertebrate brain, which is expressed by changes in the expression of neurospecific proteins (Gibbon et al., 2014).

Imidacloprid is a neonicotinoid insecticide, which is a synthetic chlorinated analogue of nicotine. This insecticide can show toxic effects in non-target organisms. Vertebrates are also exposed, but at higher concentrations of imidacloprid than invertebrates (Sera, 2005). This neonicotinoid is known to be an agonist of animal nicotinic acetylcholine receptors (nAChRs). This indicates neurotoxic properties of this insecticide (Loser, et al., 2021).

Amphibian populations may be exposed to toxic effects after imidacloprid application. There are data indicating damage to body systems and behavioural changes in tadpoles. This may further lead to impaired metamorphosis (De Arcaute et al., 2014; Pérez-Iglesias et al., 2014; Nkontcheu et al., 2017; Sweeney et al., 2021). The presence of imidacloprid in soil can affect the bioaccumulation of this substance in amphibians. This is influenced by the significant absorption properties of the amphibian skin (Van Meter et al., 2014, 2016). It is also known that imidacloprid can accumulate in amphibian tissues, including the brain of adult amphibians (Crayton et al., 2020; Campbell et al., 2022).

The functioning of the nervous system depends on the adaptive and regenerative properties of nerve cells (Hall & Tropepe, 2020). Imidacloprid is a neurotoxicant capable of causing molecular changes in animal cells (Abou-Donia et al., 2008; Huslystyi et al., 2021). The study of these phenomena may allow the identification of markers of neurotoxicity that adequately reflect critical disorders, as well as metabolic pathways that are targets for the cytotoxic compounds in a polluted environment (Manzo et al., 2001). It is known that the use of these biomarkers is possible under conditions of exposure to low concentrations of pesticides (Nedzvetsky et al., 2020; Gasso et al., 2021). Such biomarkers can be indicators of the levels of glial fibrillary acidic protein (GFAP), neurofilament light chain (NfL) and angiostatin (Abou-Donia et al., 2016; Sindi et al., 2016; Gasso et al., 2020). The possibility of using neuromolecular markers in amphi-

bians after imidacloprid exposure has not been previously investigated. Therefore, the aim of our research was to determine the effect of low concentrations of imidacloprid on the molecular parameters (GFAP, NfL, and angiostatin) of nerve cells in the brain of the adult marsh frog *Pelophylax ridibundus* Pallas, 1771.

Materials and methods

The material for the research was brain tissues taken from 30 individuals of *P. ridibundus*. The frogs were selected from ecosystems adjacent to the Dniprovsko-Orelskyi Nature Reserve. The animals were captured from late August to early September. Transportation of frogs to the laboratory took place in special cotton bags. The weight of frogs was 50.89 ± 4.21 g, length 82.62 ± 2.06 mm. Frogs were placed in a three-litre container in which the temperature was maintained at 27 °C. The containers were filled with chlorinated tap water. Acclimatization time before the experiment was three days. The volume of liquid in the containers was 1 L.

Frogs were divided into five groups, each group contained six individuals of *P. ridibundus*. Animals from the control group were kept in dechlorinated water. The exposed groups were kept in solutions with imidacloprid concentrations of 10 and 100 μg/L for 7 and 21 days. The liquid in the containers was changed every three days.

Brain tissue samples were taken after decapitation of the animals. Frog brains were washed in PBS. Brain tissue homogenization was performed in 10-fold volume of 50 mM Tris-buffer pH 7.4 (0.1 mM NaCl, 1% TritonX-100, 0.2% SDS, 2.5 mM ethylenediaminetetraacetate (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM 2-mercaptoethanol, 6.5 μM aprotinin, 1.5 μM pepstatin A, 23 μM leupeptin, 1 μM phenylmethylsulfonyl fluoride, 1 μM sodium orthovanadate, 5 μM soybean trypsin). A refrigerated centrifuge was used for centrifugation of the homogenate. The homogenate was centrifuged for 45 min at 60,000 g. The resulting supernatant was stored at –80 °C.

Western blotting (standard extraction Abcam protocol) was used to determine the expression level of GFAP, NfL and angiostatin using SDS-PAGE electrophoresis in a 5–20% acrylamide gradient with 0.1% sodium dodecyl sulphate (SDS-PAAG). Separated proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane at an electric field of 20 V/cm for 120 min (Hnasko & Hnasko, 2015; Guzyk et al., 2016). The membrane was transferred to blocking buffer with appropriately diluted antibodies. The incubation period took place overnight at 4 °C. After that, the membrane was washed with TBST and incubated in blocking buffer with diluted conjugated secondary antibodies. Then, the membrane was washed in TBST again and the results were developed by Western blotting using chemiluminescence.

Immunoblotting results were scanned and evaluated densitometrically using ImageJ software (Wayne Rasband, NIH, USA). The relative GFAP content was normalized by the total protein content in each respective sample. The study was conducted in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986) and Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty" (Revision on August 8, 2021).

Statistical analysis was performed using the non-parametric Kruskal-Wallis (K-W) test, followed by Dunn's test. The level of statistical significance was at $P < 0.05$ Statistical analyses were performed with Origin software, version 9.8 (Origin Lab Corp).

Results

The study of the content of GFAP, NfL and angiostatin in the amphibian brain was carried out to determine the specific glial and neuronal disorders caused by low and high doses of imidacloprid in comparison with the actual concentrations of imidacloprid in the contaminated sites of local ecotopes. The results of western blot showed significant differences in the content of all studied proteins (Fig. 1).

In amphibians exposed to imidacloprid at concentrations of 10 and 100 μg/L, a decrease in GFAP was determined relative to animals from the control group. After 21 days of exposure to the corresponding solutions of imidacloprid, an increase in the expression of GFAP was observed in comparison with the amphibians of the 7-days exposure, and their levelling relative to the control occurred (Fig. 2). The obtained data on the increase of GFAP expression at 21st day after its dropping at 7th day under the influence of 10 μg/L imidacloprid are statistically significant.

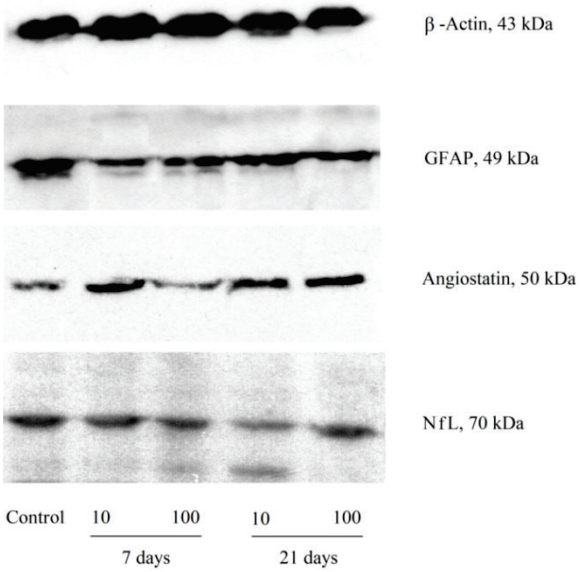

Fig. 1. Western blot for detection of GFAP, NfL and angiostatin in the brain of marsh frogs under imidacloprid (10 and 100 μg/L) exposure

The study of NfL expression showed that in *P. ridibundus* exposed to both 10 and 100 μg/L for 7 days there were no statistically significant changes in the content of this protein. On the 21st day of the experiment in frogs exposed to 10 μg/L imidacloprid, the content of NfL in the brain increased compared to the control group. However, exposure to a higher concentration of imidacloprid (100 μg/L) for three weeks leads to a significant decrease in NfL content relative to animals exposed to the same concentration for only one week. In addition, statistically significant differences were found between the groups of amphibians exposed to imidacloprid (10 and 100 μg/L) for 21 days (Fig. 3).

Significant changes also occurred in the levels of angiostatin in the brain of marsh frogs under the influence of low doses of imidacloprid. In amphibians exposed to 10 μg/L imidacloprid for 7 days and 100 μg/L for 21 days, an increase in angiostatin levels was detected relative to amphibians from the control group (Fig. 4).

We can also assert the tendency of gradual increase of angiostatin level with time under the influence of 100 μg/L imidacloprid. After 21 days of the experiment, the level of angiostatin in the amphibian brain was higher than on the 7th day.

Discussion

The results of our study showed that low concentrations of imidacloprid could cause cytotoxic changes in the brain of adult marsh frogs. After 7 days of the experiment, a decrease in GFAP in frog brain astrocytes was observed, which might indicate impaired astrocyte development (Flaskos, 2014). Given that GFAP is a classical biomarker of astrocyte disorders (Eliasson et al., 1999; Tykhomyrov et al., 2016; Shiyntum et al., 2017), a decrease in GFAP levels may indicate a dysfunction of brain nervous tissue (Müller et al., 2012). According to Markiewicz et al. (2006), a decrease in GFAP was observed in the brain of rats after administration of low doses of the pyrethroid cypermethrin after 2 and 21 days of the experiment. However, no morphological changes in astrocytes were detected, indicating the importance of molecular biomarkers. A decrease in GFAP levels was also observed in the rat cerebral cortex after administration of 100 mg/kg imidacloprid (Eser et al., 2022). In the study of Sidiropoulou et al. (2009), the metabolite of the organophosphorus insecticide diazinon (diazinon oxon, DZO) after 24 hours of exposure caused a decrease in GFAP expression in differentiated rat C6 glioma cells *in vivo*. Nevertheless, it is worth noting that in our experiment after 21 days of imidacloprid exposure the level of GFAP began to level out relative to the control.

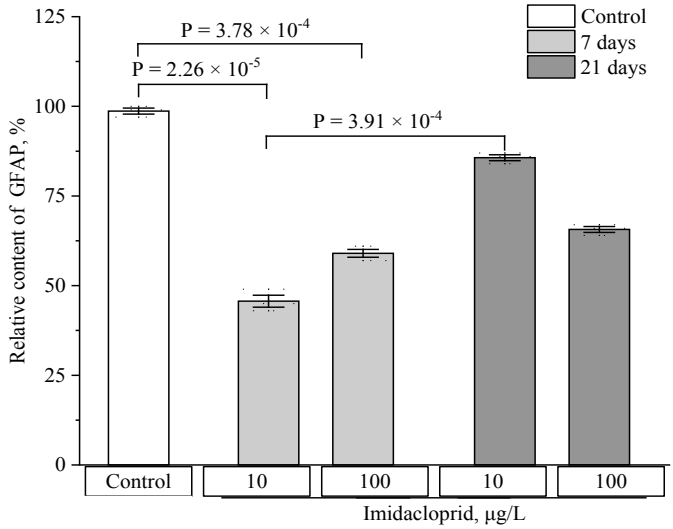

Fig. 2. Effect of imidacloprid (10 and 100 μg/L) on the GFAP level in the brain of marsh frogs

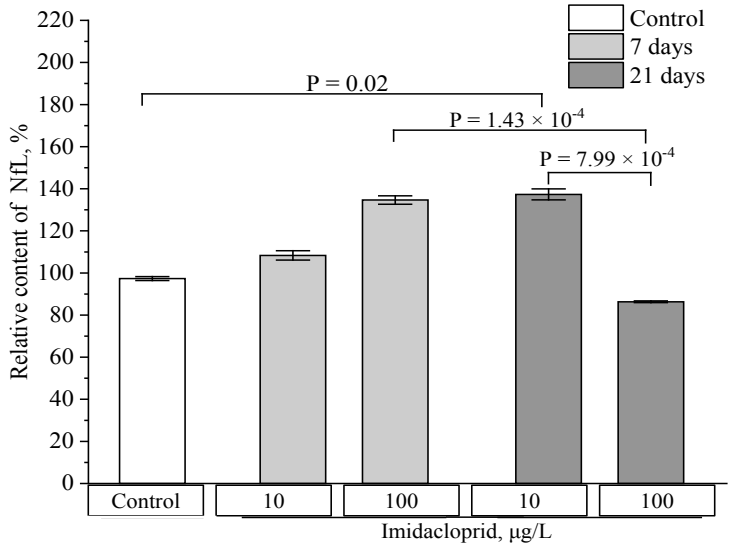

Fig. 3. Effect of imidacloprid (10 and 100 μg/L) on the NfL level in the brain of marsh frogs

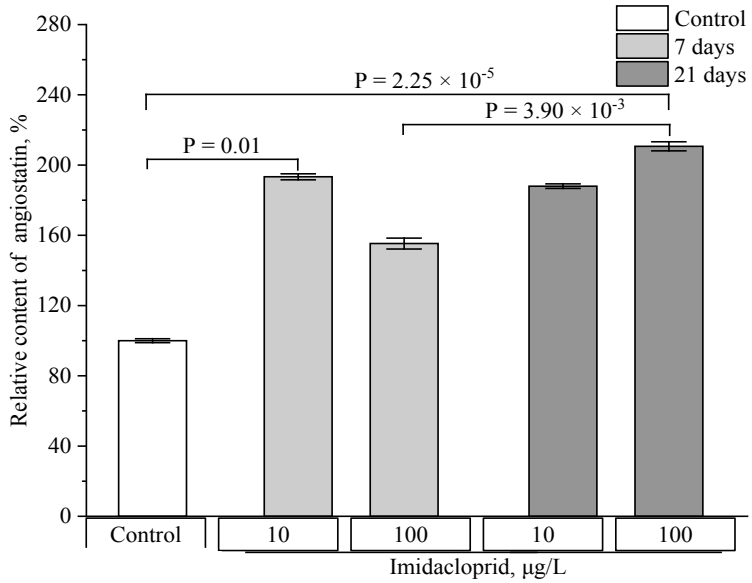

Fig. 4. Influence of imidacloprid (10 and 100 μg/L) on the angiostatin level in the brain of marsh frogs

Neurofilament light chain (NfL) is a neuronal cytoskeletal protein responsible for the development, functioning and differentiation of neuronal processes (Liem, 1993; Saunders et al., 1997; Gaetani et al., 2019). During the experiment, it was found that low concentrations of imidacloprid could affect the NfL level in the brain of adult tailless amphibians. In the animals of a group exposed for 21 days to a dose of 10 μg/L imidacloprid, a significant increase in NfL expression was found. Pandey et al. (2015) reported that cypermethrin caused an increase in NfL levels in P12 cells differentiated by neural growth factor. These proteins are biomarkers of neurodegradation as they and their fragments are products of degenerative neuronal processes (Lu et al., 2015). Increased NfL levels may indicate neuronal cytoskeletal abnormalities and cytoskeleton-related axonal transport disorders (Harmanşa & Erbaş, 2022). At the same time, there is lack of data in the literature on the effect of imidacloprid on neuronal cytoskeleton proteins. Thus, the results obtained in our study regarding the inhibition of NfL expression in the brain of adult amphibians are presented for the first time.

The results of the presented study showed that imidacloprid could affect the increase of angiostatin expression in the brain cells of adult marsh frogs. The increase in angiostatin levels was observed after 7 days at a concentration of 10 μg/L and after 21 days at a concentration of 100 μg/L, indicating the reactivity of this biomarker as a response to low doses of imidacloprid. There is a lack of studies on the effects of pesticides on angiostatin levels in the brain. Angiostatin is known to be an effective inhibitor of angiogenesis (Liang, 2016). In our work, we investigated the effect of imidacloprid on the production of angiostatin (50 kDa), which corresponds to kringle 1–5. Kringles are autonomous protein domains that are important in protein–protein interactions with blood coagulation factors. Kringle 1–5 is a product of limited proteolysis of plasminogen. The generation of kringles depends on a number of factors including the cellular response to cytotoxic factors (Tiwari, 2012). One of the important components of this cellular response is the release and activation of extracellular proteases. Some of these proteases are able to cause lysis of plasminogen and consequently generate angiostatins, in particular kringle 1–5, kringle 1–4, and kringle 2–3. All of them are capable of inhibiting angiogenesis, and thus limit the permeability of blood vessels to exogenous and toxic compounds. Thus, the increase of angiostatin detected in our study may indicate the relevant neurotoxic properties of imidacloprid.

Conclusions

Low doses of imidacloprid over a short period (1–3 weeks) cause dcertain changes in the biomarker proteins expression levels in brain cells of adult marsh frogs. Significant decrease in GFAP, a biomarker of astrocyte cytoskeleton state, was determined after 7 days of exposure relative to the control frogs. The study of NfL, which indicates neuronal cytoskeletal abnormalities and transport disorders, showed presented changes under low doses of imidacloprid exposure. The alterations also occurred in the levels of angiostatin in the marsh frog brain, which might indicate angiogenesis inhibition. Thus, low doses of imidacloprid have adverse neurotoxic effect and launch negative process in nerve cells, both neurons and astrocytes. The studied molecular biomarkers may be relevant for detection of neurotoxic effect in adult frogs under influence of neonicotinoid insecticides.

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